TRPM4 protein expression in prostate cancer: a novel tissue biomarker associated with risk of biochemical recurrence following radical prostatectomy

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Abstract: BACKGROUND Transient receptor potential cation channel, subfamily M, member 4 (TRPM4) messenger RNA (mRNA) has been shown to be upregulated in prostate cancer (PCa) and might be a new promising tissue biomarker. We evaluated TRPM4 protein expression and correlated the expression level with biochemical recurrence (BR) following radical prostatectomy (RP). MATERIAL AND METHODS The study included 614 patients who had undergone RP. TRPM4 immunohistochemical staining was performed on samples of benign tissue, tissue containing PIN glands and PCa tissue using a commercially available polyclonal antibody. Staining intensity was recorded by two independent observers using a four-tired semi-quantitative grading system (0, 1+, 2+, 3+) converted into H-scores. Interobserver agreement was calculated by linear weighted kappa statistics. The association between staining intensity and BR was analysed using the Kaplan-Meier estimator and uni- and multiple Cox proportional hazard regression models. RESULTS Significantly higher staining intensity was found in PCa glands compared to benign glands ($p < 0.001$). The concordance rate in TRPM4 staining intensities for benign, PIN and PCa tissue ranged from 86.0 to 91.5 %, corresponding to linear weighted kappa values of 0.566-0.789. After adjusting for patient and tumour characteristics, patients with a higher staining intensity in PCa glands compared to matched benign glands and an H-score equal to or above the median had an increased risk of BR (HR 1.79-2.62; $p = 0.01$-0.03 for the two observers) when compared to patients with a lower staining intensity. CONCLUSIONS TRPM4 protein expression is widely expressed in benign and cancerous prostate tissue, with highest staining intensities found in PCa. Overexpression of TRPM4 in PCa (combination of high staining intensity and a high H-score) is associated with increased risk of BR after RP.

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ORIGINAL ARTICLE

TRPM4 protein expression in prostate cancer: a novel tissue biomarker associated with risk of biochemical recurrence following radical prostatectomy

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Abstract
Background Transient receptor potential cation channel, subfamily M, member 4 (TRPM4) messenger RNA (mRNA) has been shown to be upregulated in prostate cancer (PCa) and might be a new promising tissue biomarker. We evaluated TRPM4 protein expression and correlated the expression level with biochemical recurrence (BR) following radical prostatectomy (RP).
Material and methods The study included 614 patients who had undergone RP. TRPM4 immunohistochemical staining was performed on samples of benign tissue, tissue containing PIN glands and PCa tissue using a commercially available polyclonal antibody. Staining intensity was recorded by two independent observers using a four-tired semi-quantitative grading system (0, 1+, 2+, 3+) converted into H-scores. Interobserver agreement was calculated by linear weighted kappa statistics. The association between staining intensity and BR was analysed using the Kaplan-Meier estimator and uni- and multiple Cox proportional hazard regression models.
Results Significantly higher staining intensity was found in PCa glands compared to benign glands ($p<0.001$). The concordance rate in TRPM4 staining intensities for benign, PIN and PCa tissue ranged from 86.0 to 91.5 %, corresponding to linear weighted kappa values of 0.566–0.789. After adjusting for patient and tumour characteristics, patients with a higher staining intensity in PCa glands compared to matched benign glands and an H-score equal to or above the median had an increased risk of BR (HR 1.79–2.62; $p=0.01$–0.03 for the two observers) when compared to patients with a lower staining intensity.
Conclusions TRPM4 protein expression is widely expressed in benign and cancerous prostate tissue, with highest staining intensities found in PCa. Overexpression of TRPM4 in PCa (combination of high staining intensity and a high H-score) is associated with increased risk of BR after RP.

Keywords Immunohistochemistry · Interobserver variation · Prostate cancer · Radical prostatectomy · Tissue biomarker · TRPM4

Abbreviation
BR-free survival Biochemical recurrence free survival

Institutions at which the work was performed Institute of Pathology, University Hospital Bonn (UKB), Bonn, Germany; Institute of Surgical Pathology, University Hospital Zurich (USZ), Zurich, Switzerland; Institute of Pathology, Charité University Hospital, Berlin, Germany.

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Introduction

Prostate cancer (PCa) is the most common newly diagnosed malignancy among men in western countries and is estimated to be the leading cause of male cancer deaths in Europe in 2014 only surpassed by lung cancer and colorectal cancer [1]. The course of the disease varies from entirely indolent to fatal even for organ-confined tumours [2]. Therefore, accurate and individual risk estimates based on patient and tumour characteristics are essential for treatment decisions. Although most nomograms and risk stratifications incorporate patient and tumour characteristics including prostate-specific antigen (PSA), Gleason score (GS), and clinical or pathological tumour stage (reviewed in [3]), they still have limitations in intercepting the heterogeneous course of the disease. Therefore, it is of utmost importance to identify sensitive and specific tissue, serum and urine biomarkers if the concept of personalised medicine is to become everyday clinical practice [4].

A novel group of tissue biomarkers is the transient receptor potential (TRP) channel superfamily. It consists of 28 cation-permeable transmembrane channels classified into six subfamilies: TRP canonical (TRPC), TRP vanilloid (TRPV), TRP melastatin (TRPM), TRP mucolipin (TRPML), TRP polycystin (TRPP) and TRP ankyrin (TRPA) [5]. TRP channels are activated by a variety of mechanical and chemical stimuli and are considered as polymodal sensors with impact on both physiological and pathological conditions [6].

Most but not all TRP channels function as Ca^{2+} pathways, some of which are pathways for intracellular storage and release of Ca^{2+} from various compartments (reviewed in [7]). This can lead to alterations in intracellular Ca^{2+}-levels and subsequent increased proliferation [8] and change in cell differentiation and apoptosis [9, 10]. As a consequence, differences in expression patterns of TRP channels may play a role in cancer progression [11]. The transient receptor potential cation channel, subfamily M, member 4 (TRPM4) is a nonselective cation channel activated by increased intracellular Ca^{2+} concentrations. Upon activation, Na^{+} is allowed to pass through the channel, which, in contrast, is impermeable to Ca^{2+} [12]. TRPM4 is widely expressed throughout different organs with the highest messenger RNA (mRNA) levels found in the intestine and prostate [13]. For almost a decade, it has been known that TRPM4 mRNA is upregulated in PCa compared to normal tissue [14]. However, the physiological role of TRPM4 in the prostate needs to be further elucidated. Since our own expression data showed overexpression of TRPM4 in PCa, and since others have shown that knockdown of TRPM4 in HeLa cervical cancer cells is associated with significantly decreased proliferation and a lower fraction of cells entering the S-phase of the cell cycle [15], we hypothesised that TRPM4 might also be relevant in the biology of PCa and its expression conceivable associated with prognosis.

The aim of the present study was to confirm upregulation of expression of TRPM4, which we had found in an earlier study at mRNA level, at protein expression level by immunohistochemical (IHC) staining. Furthermore, we analysed whether overexpression of TRPM4 protein is associated with an increased risk of biochemical recurrence (BR) in a large cohort of PCa patients who had undergone radical prostatectomy (RP).

Materials and methods

Patient cohort

The cohort consisted of a subset of 640 PCa patients, who underwent RP between 1999 and 2005 at the Charité University Hospital in Berlin, Germany. Twenty-six patients were excluded prior to this study due to missing tumour glands in the tissue microarray (TMA) cores or insufficient IHC staining, leaving 614 patients for analysis. The cohort and its demographics have previously been described [16] and are presented in short in Table 1. Clinical follow-up data were reviewed annually. BR was defined as postoperative PSA increase exceeding 0.2 ng/ml. The Ethics Committee of the Charité University Hospital has approved the use of the tissue for immunohistochemistry and molecular biological techniques (EA1/06/2004).

Construction of the tissue microarrays

As previously described, formalin-fixed paraffin-embedded tissue blocks from radical prostatectomy specimens were selected for construction of TMA [16]. All patients were represented by five cores, which included two cores of invasive PCa reflecting the primary and secondary Gleason pattern, if possible. Moreover, for each patient, the TMA included one core of benign prostatic hyperplasia (BPH), normal tissue and prostatic intraepithelial neoplasia (PIN), respectively. If PIN was not present in the RP specimen, another core of normal tissue was included. All cores were 1.8 mm in diameter and were arranged in 40 TMA recipient paraffin blocks.

Immunohistochemistry

Tissue microarray sections, each 3–4-μm thick, were mounted on Super Frost slides (Menzel Gläser, Braunschweig, Germany). For detection of TRPM4 expression, a commercially available antibody (goat polyclonal, G-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, catalogue no. sc-27540) was diluted 1:100. The primary antibody was detected using the refined rabbit-anti-goat protocol on the Bond

Table 1 Demographic and clinical characteristics of 614 men who underwent radical prostatectomy

	Study population n=614
Age, years, median (range); n=614	62 (43–74)
Preoperative PSA, ng/ml, median (range); n=606	7.2 (0.8–39.0)
RP specimen Gleason score, no. (%); n=614	
GS 6	219 (35.7)
GS 7	287 (46.7)
GS 8–10	108 (17.6)
Pathological tumour stage, no. (%); n=614	
pT2	426 (69.4)
pT3 / pT4	188 (30.6)
Tumour in resection margin, no. (%); n=612	
No (R-)	444 (72.5)
Yes (R+)	168 (27.5)
Preoperative hormonal therapy, no. (%); n=612	
No	569 (93.0)
Yes	43 (7.0)

GS Gleason score, *PSA* prostate specific antigen, *RP* radical prostatectomy

immunohistochemistry platform (Leica). Antibody specificity was ascertained by pre-incubation with a 10 molar excess of its corresponding peptide (TRPM4 (G-20)P; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, catalogue no. sc-27540 (peptide), which nearly completely abolished immunoreactivity (Supplementary Fig. S1).

Assessment of immunohistochemical stainings

Two independent observers (DS + KDB), both blinded to clinical parameters, biochemical outcomes and the scores of the other observer, evaluated the IHC staining. A scoring sheet depicting typical examples of staining intensities was produced after observer 1 had scored the TMA slides. Observer 2 used the identical scoring sheet to enhance the interobserver agreement.

For benign, PIN and cancerous glands, the TRPM4 staining intensity was scored separately using a four-tier semi-quantitative grading system: no staining (0), weakly positive (1+), moderately positive (2+) and strongly positive (3+). Only plasma membrane and cytoplasmic staining were considered. Furthermore, for malignant cores, the percentage of tumour cells expressing TRPM4 protein with a staining intensity of 0, 1+, 2+ and 3+ was recorded. An H-score was derived from the semi-quantitative estimates of the immunostaining: TRPM4-PCa H-score=(% of cells with staining intensity 1)+2* (% of cells with staining intensity 2+)+3*(% of cells with staining intensity 3+) [17]. Thus, the H-score range is 0 to 300, where 0 equals all tumour cells stained negative (0), whereas 300 equals all tumour cells stained strongly positive (3+). For interobserver statistical analyses, H-scores were divided into four categories: low intensity (0–50), low-moderate intensity (51–100), high-moderate intensity (101–150) and high intensity (151–300). Furthermore, patients were dichotomised according to H-scores into 'high staining intensity' (H-score equal to or above median H-score) and 'low staining intensity' (H-score bellow median H-score). To also detect subtle staining intensity differences, patients were furthermore dichotomised according to whether TRPM4 expression was higher in PCa glands compared to benign glands (TRPM4 overexpressed vs. TRPM4 not overexpressed). Equal or less staining intensity in PCa glands was reported as 'TRPM4 not overexpressed', whereas higher staining intensity in PCa glands was recorded as 'TRPM4 overexpressed'.

Statistical analysis

Associations between TRPM4 expression (high staining intensity vs. low staining intensity) and demographic and clinical variables were analysed using Pearson's chi-square test for categorical variables and the Mann-Whitney U test for continuous variables. Linear weighted Kappa statistics were used to compare interobserver agreement and Pearson's correlation was calculated to compare H-scores. The Kaplan-Meier estimator and log-rank tests were used to analyse the association between TRPM4 expression levels and risk of BR following RP. Moreover, univariate and multiple Cox proportional hazard regression models were performed with BR as outcome. Tests for linearity and proportionality were performed using cumulative and Schoenfeld residuals under the assumption that no interaction between covariates exists. Two-sided p-values <0.05 were considered significant. Statistical analyses were performed using R (R Development Core Team, Vienna, Austria, http://www.R-project.org) and SPSS version 21 (SPSS, Armonk, NY, USA).

Results

Immunoreactivity of TRPM4 in prostate tissues

TRPM4 showed a cytoplasmic expression pattern in benign and malignant tissues alike. In benign glands, basal cells were labelled (Fig. 1a), whereas secretory cells were negative. In neoplastic cells of HGPIN, TRPM4 showed stronger staining, as Fig. 1b illustrates. Epithelia of invasive tumours showed the highest degree of TRMP4 immunoreactivity. In the stroma, immunostaining of smooth muscle cells was noted but not semi-quantitatively evaluated.

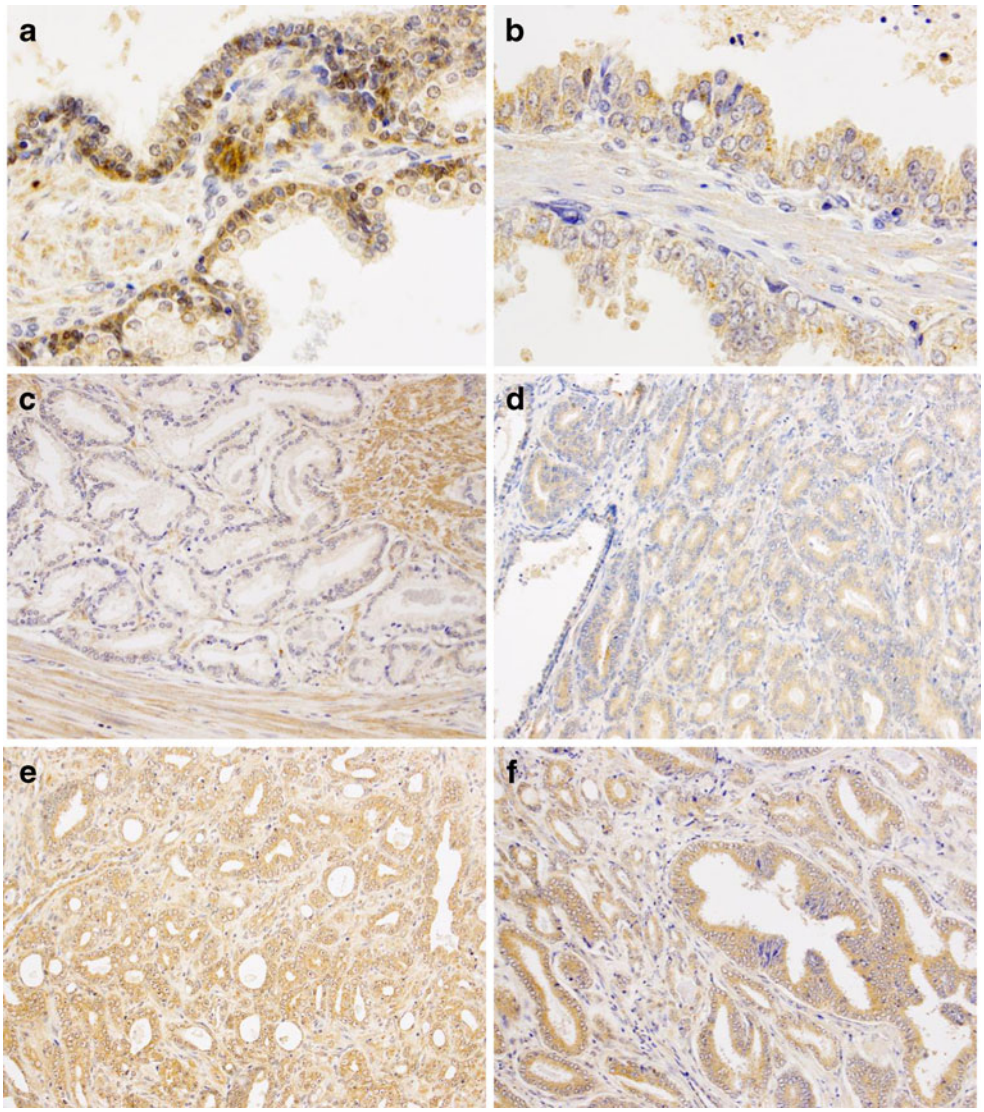

Fig. 1 TRPM4 Immunohistochemistry in prostate tissues. **a** TMPM4 is only very weakly expressed in secretory epithelium of benign glands, whereas basal cells show a weak to moderate labelling. **b** Secretory epithelium of high grade PIN glands often shows an upregulation of TRPM4 staining. **c** Prostate carcinoma with Gleason Score 3+3=6 without TRPM4 expression. Adjacent myocytes of the stroma serve as an internal positive control. **d**–f Prostate cancer with weak (**d**), moderate (**e**) or strong (**f**) TRPM4 positivity

Interobserver variation in TRPM4 intensity scoring

All 614 patients had valid TRPM4 intensity scores for benign and cancerous tissue cores from both observers, whereas 386 patients had two valid PIN TRPM4 intensity scores. The interobserver concordance was 89.3, 91.5 and 86.0 % for benign, PIN and tumour TRPM4 intensity, respectively, corresponding to linear weighted Kappa values of 0.566 (95 % CI 0.513–0.620), 0.789 (95 % CI 0.703–0.875) and 0.744 (95 % CI 0.682–0.806) (Table 2). When H-scores were divided into four groups, the concordance between the observers was 60.1 % corresponding to a linear weighted Kappa value of 0.541 (95 % CI 0.489–0.592). There was a significant and strong correlation between the two H-scores when analysed on a continuous scale (Pearson's correlation coefficient=0.775, $p<0.001$).

TRPM4 protein expression in prostate cancer

Demographics and tumour characteristics of the patient population has previously been published [16] and are listed in Table 1. In total, 43 patients (7.0 %) had received gonadotropin-releasing hormone analogues before surgery at the discretion of the referring urologist. Median time of treatment was 4 weeks (range 2–16 weeks).

The predominant TRPM4 intensity was 1+ for benign glands (84.4–89.3 %), PIN (73.4–76.4 %) and PCa glands (63.0–66.8 %) for both observers (Figs. 1 and 2). However, statistically significantly higher TRPM4 staining intensity was found in PCa glands compared to benign tissue and PIN (chi-square test for trend 50.81 and 126.54, respectively; 1 df; both $p<0.001$). In cancerous glands, a predominant TRPM4 intensity of 2+ and 3+ was found in 25.6–30.8 and 1.6–2.0 %, respectively. Reflecting that the predominant TRPM4

Table 2 Interobserver agreement on immunohistochemical staining intensity of TRPM4

Benign TMA cores								
		Observer 2						
		0	1+	2+	3+	Patients eligible	Concordance	Weighted Kappa
						n=614	89.3 %	0.566
Observer 1	0	20	3	0	0			(95 % CI 0.513–0.620)
	1+	36	500	12	0			
	2+	0	15	28	0			
	3+	0	0	0	0			
PIN TMA cores								
		Observer 2						
		0	1+	2+	3+	Patients eligible	Concordance	Weighted Kappa
						n=386	91.5 %	0.789
Observer 1	0	6	3	0	0			(95 % CI 0.703–0.875)
	1+	0	276	23	0			
	2+	0	6	68	0			
	3+	0	0	1	3			
PCa TMA cores								
		Observer 2						
		0	1+	2+	3+	Patients eligible	Concordance	Weighted Kappa
						n=614	86.0 %	0.744
Observer 1	0	26	10	1	0			(95 % CI 0.682–0.806)
	1+	0	359	51	0			
	2+	0	18	135	4			
	3+	0	0	2	8			
PCa TMA cores–H-scores								
		Observer 2						
		0–50	51–100	101–150	151–300	Patients eligible	Concordance	Weighted Kappa
Observer 1	0–50	35	35	11	0	*n*=614	60.1 %	0.541
	51–100	8	170	102	24			(95 % CI 0.489–0.592)
	101–150	1	12	94	38			
	151–300	0	0	14	70			

intensity was 1+, the median H-score was 100 (range 0–300) and 110 (range 5–290) for observers 1 and 2, respectively, with interquartile ranges of 100 to 150 and 80 to 150.

No association between TRPM4 expression and patient or tumour characteristics (age, PSA, GS, pathological tumour stage, and resection margin status) was found (Table 3).

TRPM4 protein expression and biochemical recurrence

PSA relapse-free survival time was available for 576 patients (93.8 %). When dichotomising patients into 'TRPM4 overexpressed' vs. 'TRPM4 not overexpressed', there was no significant difference between the two groups in terms of risk of BR for the two observers (log-rank: p=0.05 and p=0.66, respectively) (Supplementary Fig. S2a + b). Although only statistically significant for observer 2, patients with 'high staining intensity' had an increased risk of BR compared to patients with 'low staining intensity' (log-rank: p=0.061 and p=0.015, respectively) (Supplementary Fig. S2c + d).

A combined three-tiered variable was created representing intrapersonal and interpersonal differences in TRPM4 expression. Patients were characterised as either 'TRPM4 not overexpressed and H-score below median', or 'TRPM4 overexpressed or H-score equal to or above median', or 'TRPM4 overexpressed and H-score equal to or above median'. On the records of observer 1, the groups separated into three different courses of BR (log-rank: p=0.043), with patients characterised as 'TRPM4 overexpressed and H-score equal to or above median' having the shortest BR free survival (Fig. 3a). For observer 2, the 'TRPM4 not overexpressed and H-score below median' group showed the best BR free survival (Fig. 3b), whereas the two other groups overlapped. Correspondingly, no significant difference in disease course was observed between the groups (log-rank: p=0.15).

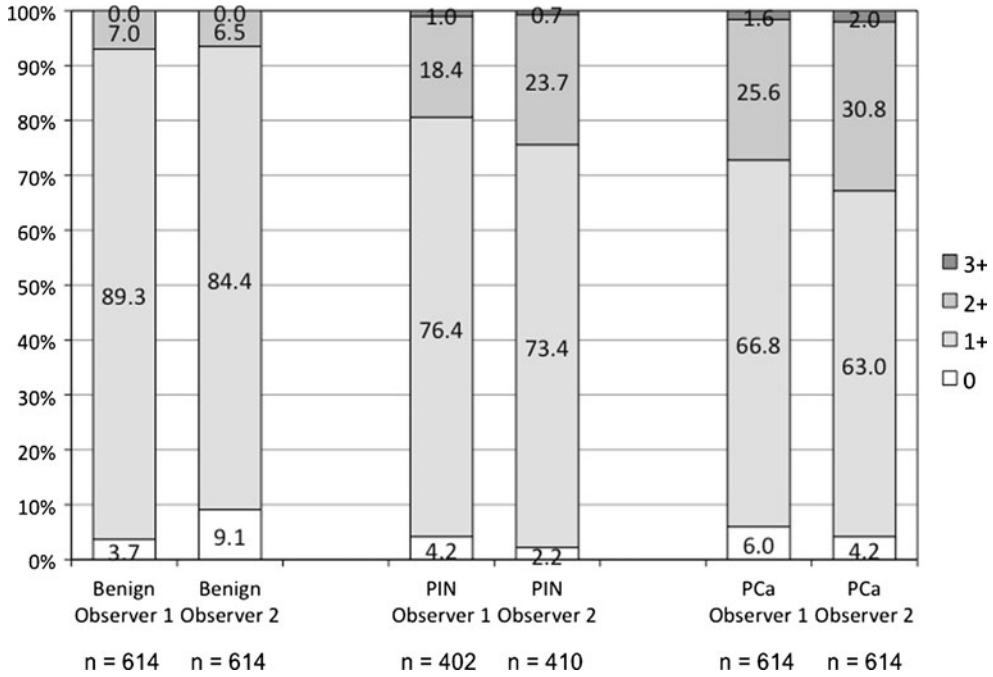

Fig. 2 TRPM4 expression in prostate tissues at protein level. Illustration of the progression of TRPM4 expression from normal tissue through PIN to invasive carcinoma. Immunohistochemical data from observer 1 (*left-most columns*) and observer 2 (*right-most columns*)

TRPM4 was entered into two univariate and multiple Cox proportional hazard regression models (model A and B) using the individual TRPM4 sum scores for observer 1 and 2, respectively. In both models, TRPM4 overexpression in combination with high staining intensity (i.e. TRPM4 overexpressed in PCa compared with benign tissue combined with an H-score equal to or above median) was associated with an increased risk of BR (Table 4). This remained statistically significant after adjusting for age, PSA, GS, pathological tumour stage, surgical margins and prior hormonal treatment (model A: HR 2.62; 95 % CI 1.26–5.42; p=0.010; model B: HR 1.79; 95 % CI 1.06–3.04; p=0.030).

Discussion

This is the first report on TRPM4 protein expression in prostate tissues using a large, well-characterised cohort of PCa patients who have undergone RP. Our group has previously shown that TRPM4 mRNA is among the top upregulated transcripts in PCa [14], which is in line with other profiling studies [18–20]. In the present paper, we used IHC to detect TRPM4 protein and found that it is commonly expressed in prostatic tissue. Protein expression was predominantly low, although we demonstrated a significant trend for higher semi-quantitative expression levels in PCa tissue compared to benign and PIN glands. Although high TRPM4 expression as such was not found to be associated with BR, the combination of having high TRPM4 in PCa compared to matched benign tissue with an H-score equal to or above median appeared to be a strong predictive marker for BR. This corresponded to a 1.8–2.6-fold increased risk of BR after RP when compared to patients without high TRPM4 expression and a low H-score. Interestingly, we found no associations between TRPM4 expression and tumour characteristics, indicating that TRPM4 is an independent prognostic marker. This needs to be verified in future studies to clarify if TRPM4 might be a useful candidate as prognostic test for prostate cancer, which clearly cannot be based on a single retrospective study.

The TRPM4 protein consists of six transmembrane domains and forms a nonselective cation channel after assembly of four subunits upon activation, although the stoichiometry is not yet fully known (reviewed in [12]). The channel is activated by Ca^{2+} [21–23], has the highest permeability for Na^{+} and K^{+}, and is practically impermeable to Ca^{2+} and Mg^{2+} [21, 23, 24]. It has previously been demonstrated that TRPM4 mRNA is widely expressed in numerous types of tissue, the highest mRNA transcript levels being found in the prostate and the intestines [13]. Whereas the physiological function of TRPM4 in the prostate is unknown, it is involved in such diverse functions as depolarisation of vascular smooth muscle cells [25], regulation of sinus rhythm of the heart [26] and the process of cell death through Na^{+} influx [27]. Furthermore, TRPM4 expression is upregulated in an aggressive form of large B-cell non-Hodgkin lymphoma [28], and high expression has been found to correlate with tumour progression and metastatic disease [18, 28].

In a recent study by Armisén et al., knockdown of TRPM4 with shRNA in HeLa cervical cancer cells was shown to significantly decrease proliferation, increase the fraction of cells in the G1 phase of the cell cycle and decrease the fraction of cells

Table 3 Demographic and clinical characteristics of 614 men who underwent radical prostatectomy stratified on TRPM4 staining intensity

Observer 1	Low staining intensity $n = 124$	High staining intensity $n = 490$	p – value*
Age, years, median (range)	63 (45 – 73)	62 (43 – 74)	0.74
Preoperative PSA, ng/ml, median (range)	7.3 (2.4 – 30.4)	7.2 (0.8 – 39.0)	0.20cxx
RP specimen Gleason score, no. (%)			0.58
GS 6	40 (32.3)	179 (36.5)	
GS 7	63 (50.8)	224 (45.7)	
GS 8 – 10	21 (16.9)	87 (17.8)	
Pathological tumour stage, no. (%)			0.27
pT2	81 (65.3)	345 (70.4)	
pT3 / pT4	43 (34.7)	145 (29.6)	
Tumour in resection margin, no. (%)			0.86
No (R-)	90 (73.2)	354 (72.4)	
Yes (R+)	33 (26.8)	135 (27.6)	
Observer 2	Low staining intensity $n = 292$	High staining intensity $n = 322$	
Age, years, median (range)	63 (43 – 73)	62 (43 – 74)	0.99
Preoperative PSA, ng/ml, median (range)	7.1 (1.0 – 39.0)	7.3 (0.8 – 38.0)	0.82
RP specimen Gleason score, no. (%)			0.32
GS 6	112 (38.4)	107 (33.2)	
GS 7	134 (45.9)	153 (47.5)	
GS 8 – 10	46 (15.8)	62 (19.3)	
Pathological tumour stage, no. (%)			0.67
pT2	205 (70.2)	221 (68.6)	
pT3 / pT4	87 (29.8)	101 (31.4)	
Tumour in resection margin, no. (%)			0.40
No (R-)	215 (74.1)	229 (71.1)	
Yes (R+)	75 (25.9)	93 (28.9)	

entering the S phase [15]. The authors further demonstrated that knockdown of TRPM4 results in decreased expression of cyclin D1, which is essential for the transition from G1 to S phase, decreased expression of survivin, a protein involved in progression of mitosis and resistance to apoptosis, as well as a decreased expression of β-catenin. In the absence of Wnt signalling, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3β for degradation, whereas Wnt signalling promotes translocation of β-catenin to the nucleus, which transactivates various genes associated with proliferation and cell survival (reviewed in [29, 30]). Indeed, Armisén et al. found that β-catenin is primarily located in the cytoplasm after TRPM4 knockdown, compared to its nuclear location in TRPM4 expressing HeLa cells. The authors conclude that TRPM4 exerts its effects through inhibition of degradation of β-catenin, leading to nuclear translocation and increased cell proliferation [15]. In line with our results, this suggests that a high level of TRPM4 in a cancer is associated with a more aggressive course.

Most changes involving TRP proteins is caused by increased or decreased expression of the normal protein rather than mutations, and several members of the TRP superfamily show altered expression in cancer cells [11]. Previous studies on PCa have mainly focused on TRPM8 and TRPV6. Although the published results are conflicting, significantly higher expression levels of TRPM8 mRNA have been found in PCa in comparison to benign tissue [31]. However, higher TRPM8 levels were found to be inversely correlated to GS and tumour stage, although not statistically significant [32]. Additionally, low levels of TRPM8 mRNA expression were associated with a significant shorter time to BR following RP [33]. Hence, it seems as if expression level of TRPM8 is higher in PCa compared to benign tissue, but lower in aggressive PCa. For TRPV6, normal epithelial cells and BPH tissue show mRNA expression, but interestingly, a higher expression level is found in PCa tissue with a positive correlation with GS and pathological tumour stage [34, 35].

The present study has several limitations. First, we used BR as the endpoint in the Kaplan-Meier and the Cox proportional

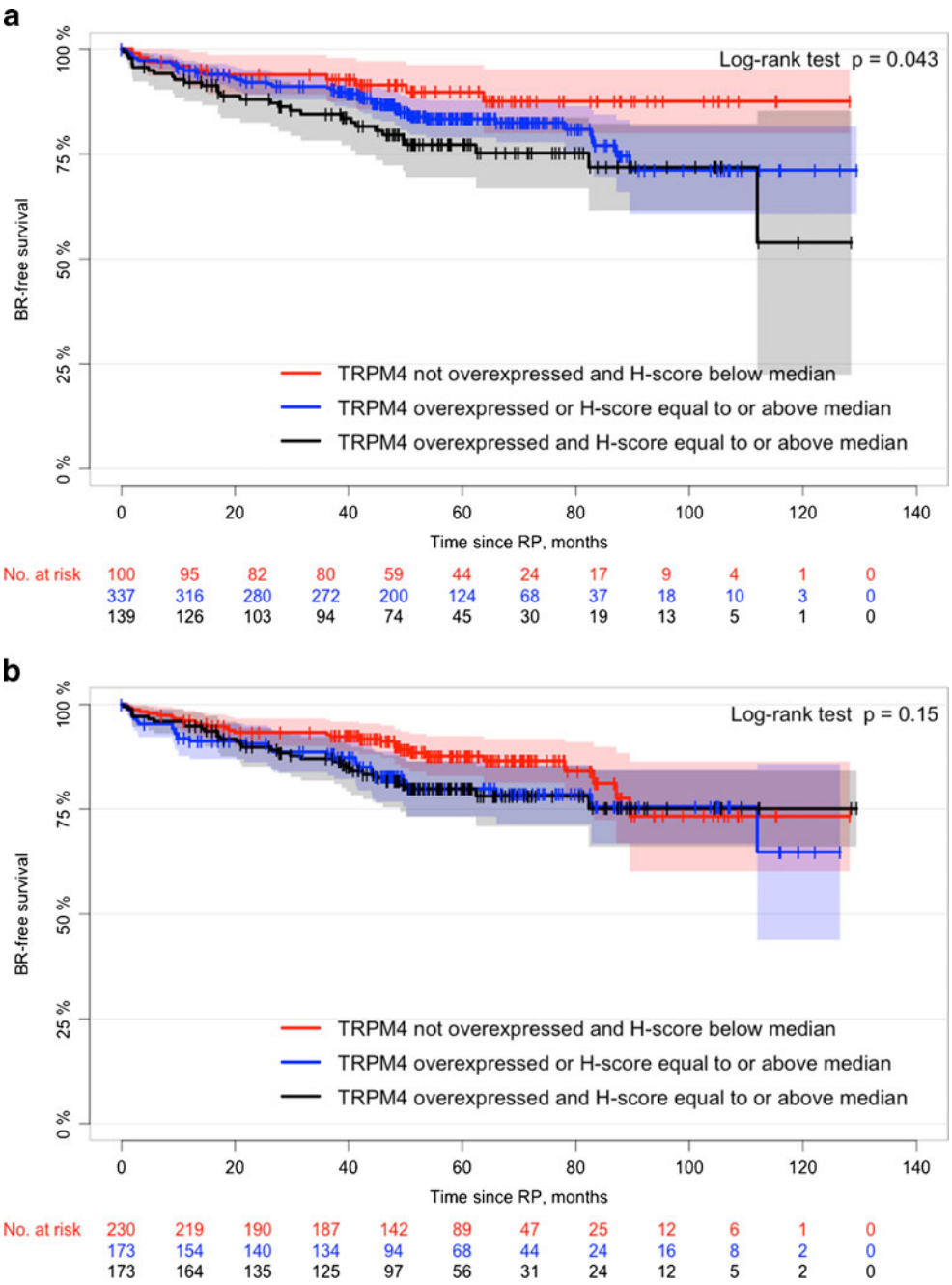

Fig. 3 Kaplan-Meier estimated biochemical recurrence free survival curves for 576 patients with available biochemical follow-up data. **a** Patients are stratified into three groups according to observer 1's records on TRPM4 overexpression in PCa compared to benign tissue and H-scores. **b** Patients are stratified into three groups according to observer 2's records on TRPM4 overexpression in PCa compared to benign tissue and H-scores. *BR-free survival* biochemical recurrence free survival

hazard regression analyses. Although BR is a widely used surrogate endpoint, it is well-known that a large proportion of patients will not develop symptoms, metastasis let alone PCa-related death even 10–15 years after BR, even if they do not receive adjuvant or salvage therapy [36]. Secondly, in most patients, PCa is a multifocal disease, and both intrafocal and interfocal heterogeneity are critical issues and limitations when studying tissue biomarkers, i.e. the accuracy of the IHC results might be limited by variations in expression patterns within and between tumour foci. Indeed, intrafocal and interfocal staining heterogeneity for TRPM4 was observed for up to 97 % of the patients in the present study. To minimise the impact of this heterogeneity, we used two TMA cores per patient reflecting the primary and secondary Gleason pattern, respectively, and calculated an H-score for each patient. It might be argued, however, that two cores is insufficient to fully elucidate heterogeneity [37]. Thirdly, semi-quantitative IHC grading will inherently result in interobserver variation [38]. Before introducing a new biomarker, it is essential to acknowledge this drawback and moreover comprehend that semi-quantitative grading is affected by subjectivity and indeed is only semi-quantitative. TRPM4 expression is not specific to epithelial cells but was also observed in stromal cells, which can impede accurate reading and might negatively

Table 4 Univariate and multiple Cox proportional hazard regression models for risk of biochemical recurrence after radical prostatectomy

	Univariate analysis			Multiple analysis		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
Model A						
Age, 5 year change	0.89	(0.75-1.06)	0.20	0.84	(0.70-1.00)	0.055
PSA, 2-fold change	1.55	(1.17-2.06)	0.002	1.21	(0.91-1.60)	0.20
Gleason score						
GS 6	1	(ref)		1	(ref)	
GS 7	2.61	(1.42-4.77)	0.002	2.16	(1.15-4.08)	0.017
GS 8 – 10	6.62	(3.58-12.21)	<0.001	3.16	(1.56-6.40)	<0.001
Pathological tumour stage						
pT2	1	(ref)		1	(ref)	
pT3 / pT4	4.62	(3.02-7.06)	<0.001	3.18	(1.94-5.21)	<0.001
Tumour in resection margin						
No	1	(ref)		1	(ref)	
Yes	2.90	(1.93-4.36)	<0.001	1.39	(0.86-2.23)	0.18
Preoperative hormonal therapy						
No	1	(ref)		1	(ref)	
Yes	1.59	(0.82-3.06)	0.17	1.38	(0.71-2.67)	0.34
TRPM4 expression and staining intensity						
Not overexpressed and Low	1	(ref)		1	(ref)	
Overexpressed or High	1.67	(0.85-3.27)	0.14	1.57	(0.79-3.12)	0.20
Overexpressed and High	2.38	(1.16-4.87)	0.017	2.62	(1.26-5.42)	0.010
Model B						
Age, 5 year change	0.89	(0.75-1.06)	0.20	0.84	(0.70-1.00)	0.053
PSA, 2-fold change	1.55	(1.17-2.06)	0.002	1.20	(0.90-1.58)	0.21
Gleason score						
GS 6	1	(ref)		1	(ref)	
GS 7	2.61	(1.42-4.77)	0.002	2.11	(1.12-3.97)	0.022
GS 8 – 10	6.62	(3.58-12.21)	<0.001	3.11	(1.54-6.26)	0.002
Pathological tumour stage						
pT2	1	(ref)		1	(ref)	
pT3 / pT4	4.62	(3.02-7.06)	<0.001	3.26	(1.99-5.34)	<0.001
Tumour in resection margin						
No	1	(ref)		1	(ref)	
Yes	2.90	(1.93-4.36)	<0.001	1.39	(0.86-2.24)	0.18
Preoperative hormonal therapy						
No	1	(ref)		1	(ref)	
Yes	1.59	(0.82-3.06)	0.17	1.30	(0.67-2.52)	0.43
TRPM4 expression and staining intensity						
Not overexpressed and Low	1	(ref)		1	(ref)	
Overexpressed or High	1.55	(0.94-2.56)	0.085	1.42	(0.85-2.39)	0.18
Overexpressed and High	1.54	(0.93-2.55)	0.092	1.79	(1.06-3.04)	0.030

The two models include different evaluations of TRPM4 staining. Model A includes a combined variable representing both TRPM4 expression in PCa compared to benign glands and the staining intensity for observer 1 ('not overexpressed and low staining intensity' vs. 'overexpressed or high staining intensity' vs. 'overexpressed and high staining intensity'). Model B includes the same combined variable for observer 2. High indicates TRPM4 staining intensity equal to or above median H-score. Low indicates TRPM4 staining intensity bellow median H-score. Not overexpressed indicates staining intensity in prostate cancer glands equal to or bellow benign glands. Overexpressed indicates staining intensity in prostate cancer glands above benign glands

GS Gleason score, *PSA* prostate specific antigen, *pT* pathological tumour stage, *ref* reference

influence interobserver agreement. In the present study, we attempted to minimise the interobserver variation by creating a TRPM4 scoring sheet, which resulted in approximately 90 % concordance rates indicating moderate to substantial agreement according to the weighted kappa values [39]. Finally, this study lacks an independent validation cohort to confirm the potential of TRPM4 as a prognostic biomarker, which was, however, not the primary aim of this study. Strengths of the present study are the large well-characterised cohort of PCa patients, the long-term follow-up and the inclusion of two independent observers.

TRP channels might be targets for management of cancer using potent channel inhibitors or using the TRP proteins as recognition sites for antibody-mediated toxic entry (reviewed in [11]). While the channel might be a suitable and attractive pharmacological target, blocking the channel might lead to serious adverse events due to the wide distribution of the protein. Indeed, TRPM4 knockdown mice are characterised by mild hypertension, increased risk of anaphylaxis and increased risk of death in case of sepsis [12]. Thus, at the moment, TRPM4 does not seem to be a suitable pharmacological target for management of PCa but seems to provide important prognostic information in terms of risk of BR following RP.

Conclusions

We show that TRPM4 protein is widely expressed in benign prostate tissue, PIN glands and cancerous glands with highest scoring intensities in PCa. TRPM4 expression can be reliably visualised using IHC staining with a commercial available polyclonal antibody. Moreover, the use of a scoring sheet resulted in substantial agreement in TRPM4 staining intensity scores between two observers. Finally, the combination of TRPM4 overexpression in PCa in comparison to matched benign glands and an H-score equal to or above the median was independently associated with an increased risk of BR following RP, which warrants further study.

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Compliance with ethical standard

Financial disclosure/Conflict of Interest None.

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